AD_____

COOPERATIVE AGREEMENT NUMBER DAMD17-96-2-6017

TITLE: A Novel Gene Gun-Mediated IL-12 Gene Therapy for Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. Ning-Sun Yang

CONTRACTING ORGANIZATION: PowderJect Vaccines, Inc.
Madison, WI 53711

REPORT DATE: January 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 1999	Annual (15 Sep 97-14 Sep 98)

4. TITLE AND SUBTITLE
A Novel Gene Gun-Mediated IL-12 Gene Therapy for Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-2-6017

6. AUTHOR(S)
Dr. Ning-Sun Yang

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Powderject Vaccines, Inc.
585 Science Drive, Madison, WI 53711

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The overall goal of our research is to develop an immunological approach for breast cancer gene therapy. The results of the first year study, described in our previous Annual Report, show that gene gun-mediated IL-12 gene therapy is effective against breast tumors in mouse models. During the second year of this study we demonstrated that 4T1 tumor is weakly immunogenic, and it can induce a low level immune response. However, the anti-metastatic effect of IL-12 gene therapy against 4T1 tumor is not mediated by T cells, but rather involves NK cells. From several different immunomidulatory genes tested in combination with IL-12 gene therapy, IL-18 and ICE were found to be more effective in treatment of established TS/A breast tumor than IL-12 alone.

In a separate, but strategically relevant approach for cancer gene immunotherapy, a striking antitumor effect was demonstrated in mice vaccinated with a gene encoding the tumor-associated antigen gp100 in combination with a GM-CSF gene. The results of this study strongly suggest that gene gun-mediated *in vivo* IL-12 gene therapy approach in combination with other immunological approaches may be developed as an effective and safe alternative to systemic IL-12 protein for treatment of breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	34
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

NSV Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

NSV In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

NSV For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NSV In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

NSV In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

___ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

N. E. Y. Jan 27, 1999

PI - Signature Date

TABLE OF CONTENTS

A. INTRODUCTION

Invasive breast cancer is the leading form of cancer among women and the second biggest killer, after lung cancer (1). For a woman living in North America, the lifetime odds of getting breast cancer now stand at 1 in 8, double the risk of 1940 (1). Approximately 180,000 women were expected to be diagnosed with breast cancer in 1995 (2), with more than 40,000 expected to die from metastatic breast cancer (3).

Although substantial progress has been made in the detection and treatment of localized (nonmetastatic) disease, there has been relatively modest progress in the treatment of advanced disease. Thus, there is an urgent need for new, effective therapeutic approaches for metastatic breast cancer.

Cytokine therapy is regarded by many clinical investigators as one of the more promising approaches for treatment of advanced forms of cancers, including breast cancer, because it can be directed at eradication of both the primary tumor and its metastases via activation of an antitumor immunity. Among various cytokines, IL-12 in particular exerted dramatic antitumor effects in several different experimental tumor models (4,5). Unfortunately, little information is available for mammary tumor models. In addition, recombinant IL-12 protein in therapeutic doses can be toxic to mice and humans (6,7).

The ultimate goal of our research is to develop an immunological approach for breast cancer gene therapy that can result in regression of both primary tumors and residual metastatic foci, and can also induce sufficient immunological memory to prevent tumor recurrence and progression. Based upon our previous gene gun studies, this strategy is expected to exploit the gene therapy potential for treatment of breast cancer without the toxic side effects encountered in other studies employing cytokine protein therapy (6,7).

We recently reported (8,9) that gene gun-mediated *in vivo* delivery of IL-12 DNA elicited effective antitumor responses with no evident toxicity. This therapeutic effect was achieved via localized transgenic production of IL-12 protein, at a systemic level at least 1,000 times less than the effective, and toxic, dose of recombinant IL-12 protein delivered systemically (6,7,10). We therefore anticipated that gene gun-mediated IL-12 gene therapy might be effective and non-toxic in murine models of mammary cancer.

Our previously published study showed that six out of six tumor models tested, including two sarcomas, a renal cell carcinoma, a lymphoma, a melanoma and a mastocytoma, responded at varying degrees to gene gun-mediated IL-12 gene therapy *in vivo* (8). Responses varied from complete regression to a significant suppression of tumor growth, depending on the immunogenicity of test tumors. These syngeneic mouse tumor models resemble several currently employed murine mammary tumor models, for which non-immunogenic, poorly immunogenic, and highly immunogenic tumor cell lines have been characterized, and histology, tumorigenicity, and metastatic capacity recently established (11-17). Thus there was good reason to suggest that murine mammary tumors also may also be responsive to this gene therapy protocol.

Indeed, results obtained from our first year study, described in our previous Annual Report, show that gene gun-mediated IL-12 gene therapy is effective against breast tumors in mouse models. The results obtained by the beginning of the second year of this study can be summarized as follows:

- The TS/A mammary adenocarcinoma is moderately immunogenic, whereas the 4T1 mammary adenocarcinoma is apparently non-immunogenic.

- IL-12 gene therapy of the immunogenic TS/A adenocarcinoma results in complete regression of 50% of the established primary tumors and induction of immunological memory.

- IL-12 gene therapy of the non-immunogenic 4T1 adenocarcinoma does not significantly affect the growth of the primary tumor, but reduces metastasis into the lungs.

- A brief course of IL-12 gene therapy significantly extends mouse survival time following the excision of a 4T1 primary tumor.

- The anti-metastatic effect of IL-12 gene therapy against the 4T1 tumor appears to be not T cell-mediated.

During the second year of this study we further characterized the antitumor and anti-metastatic effects of IL-12 gene therapy against breast tumors. We focused mainly on two aspects: 1) Characterize the role of systemic immunity, T cells, and NK cells in the observed anti-metastatic effect against 4T1 tumors, and 2) Develop new experimental protocols for augmenting the antitumor effect of IL-12 gene therapy. The results obtained to date have been quite encouraging, and are described as follows.

B. BODY OF REPORT

B1. Experimental Methods

Mice: Balb/c mice were obtained from Harlan-Sprague Dawley (Madison, WI), and Balb/c nude mice from Taconic (Germantown, NY). Female mice 8-12 weeks of age were used in the experiments. Housing, care and use of mice were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, National Institutes of Health, Bethesda, MD, 1985).

Mammary tumor models: TS/A adenocarcinoma (11,17) and 4T1 adenocarcinoma (13) were kindly provided by G. Forni (Immunogenetic and Histocompatibility Center, Turin, Italy) and F.R. Miller (Michigan Cancer Foundation, Detroit, MI), respectively. Both cell lines were established from spontaneous, moderately differentiated mammary adenocarcinomas growing in Balb/c mice and are metastatic. Tumor cell cultures were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and gentamycin at 50 μg/ml. Before being injected into mice, tumor cells were detached from the plastic by a short incubation in Trypsin-EDTA solution. Mice were shaved in the abdominal area and injected intradermally (i.d.) with $1x10^5$ viable tumor cells in 50 μl Dulbecco's Phosphate Buffered Saline. For most *in vivo* experiments, 8 mice per group were tested; for survival experiments, 12-16 mice per group were evaluated. Growth of primary tumor was monitored 2-3 times a week by measuring two perpendicular tumor diameters using calipers. Tumor metastasis was estimated by weighing the excised lungs on day 31-38 after tumor cell implantation, when it was noted that control mice had become moribund.

Transgene expression vectors: We used plasmid pWRG3169, which contains the coding sequences for the p35 and p40 subunits of murine IL-12 arranged in the same orientation in tandem, with each driven by its own CMV promoter. This construction gave us the highest level of IL-12 functional activity (stimulation of ConA-activated spleen cells) and immunoreactivity (by ELISA) when compared with other molecular arrangements, including a bi-cistronic construct with an internal ribosome entry site. This and a luciferase (Luc) cDNA expression vector have been described previously (8).

***In vivo* IL-12 gene transfer via gene gun:** For all *in vivo* skin transfection experiments, we utilized a helium-pulse Dermal PowderJect-XR (formally *Accell*®) gene gun (Powderject Vaccines, Inc., Madison, WI) as previously described (8). Briefly, plasmid DNA was precipitated onto gold particles (2.1 μm in diameter) and coated onto the inner surface of a Tefzel tubing (1/8" outside diameter, 0.93" inside diameter; McMaster-Carr, Chicago). The tubing was cut into half-inch segments, each segment being a gene delivery "cartridge". Conditions were adjusted so that each cartridge contained 0.5 mg of gold and 1.25 μg of plasmid DNA. For *in vivo* gene delivery, mouse skin overlying and surrounding the target tumor was transfected with either the IL-12 cDNA or the Luc cDNA expression vector. Each treatment consisted of four transfection shots (i.e., 4 cartridges were used per treatment), containing a total of 5 μg of plasmid DNA. The DNA-coated gold particles were propelled by a helium gas pulse, at a discharge pressure of 300 pounds per square inch (psi). One transfection shot was delivered

directly over the tumor site, and three additional transfection shots were placed at adjacent sites around the tumor site. This gene therapy procedure was performed 2-3 times per week for two weeks.

***In vivo* cell depletion assays**: To deplete T cells, a mixture containing 300 μg of each anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43) was administered intraperitoneally in 4-5 day interval. Flow cytometric analysis of peripheral blood cells of these mice, performed 4 days after the last mAb injection, confirmed depletion of 98% of $CD3^+$ cells. Control mice received Rat IgG (Sigma) at the dose 600 μg/mouse per day following the same schedule as the anti-CD4/CD8 mAbs. To deplete NK cells, mice were injected via tail vein with 40 μl of anti asialo GM1 antibody (Wako BioProducts, Richmond, VA) diluted in PBS according to manufactor's recommendations. Mice received Ab treatments on day 6, 10 and 15 post tumor cell implantation. Control mice received the equivalent dose (1.5 mg/mouse) of Rabbit IgG (Sigma, St.Louis, MO).

***In vitro* cellular immunology assays:** Experimental mice were treated with IL-12 cDNA or Luc cDNA on days 7 and 10 post 4T1 tumor cell implantation, and tumor draining (axillary) lymph nodes (TDLN) were taken on day 11. Lymph node cells ($2x10^6$) were incubated in culture medium without any stimuli at 37°C for 48 hr. Cell culture supernatants were collected and frozen at -20°C until testing. IFNγ levels in cell culture supernatants were measured by an ELISA kit (Endogen, Cambridge, MA). Activity of natural killer NK) cells was determined in a standard 4-hr ^{51}Cr release assay using NK-sensitive YAC-1 cells as targets.

B2. Results

Analysis of immunogenicity of TS/A and 4T1 tumors.

Since human breast cancer, like most of the other types of cancer, is considered to be poorly immunogenic (18), we employed for our study TS/A adenocarcinoma, which was described as non-immunogenic in some studies (11) and as poorly immunogenic in later studies by the same authors (19). We also used the 4T1 tumor, which was personally communicated as non-immunogenic by Dr. F. Miller (Michigan Cancer Foundation, Detroit, Michigan). The results of our first immunogenicity experiment, described in previous Annual report, suggested that TS/A is an immunogenic tumor, and 4T1 is apparently non-immunogenic. Since it is known that immunogenicity of a tumor may depend on the dosage and design of immunization or tumor challenge, we decided to perform a more detailed analysis of the immunogenicity of TS/A cells. Indeed, the results of two additional experiments show that when 10^5 4T1 tumor cells were used for a challenge, the protective effect of vaccination was not detected. However, the immune response could be detected when 10^4 or less tumor cells were used for challenge (**Figure 1**). Therefore, the 4T1 tumor can be classified as "weakly immunogenic", rather than "non-immunogenic". We have also obtained additional evidence confirming that low level immune response can be generated against the 4T1 tumor. Specifically, when mice immunized against TS/A tumors were challenged intradermally with 4T1 tumor several weeks later, a significantly reduced tumor growth was observed as compared with naïve mice challenged with the same dose of 4T1 cells. These results suggest that TS/A tumor and 4T1 tumor express shared tumor antigens, and that the immune response to 4T1 tumor can be induced if the immunogen is strong enough.

Increasing immunogenicity of 4T1 cells.

Tumor vaccination utilizing tumor cells transfected with cytokine genes (e.g. IFN-γ) has been shown to be a promising *ex vivo* approach (16). We hypothesized that *ex vivo* modification of a poorly immunogenic 4T1 tumor with IFN-γ gene transfection may make this tumor more immunogenic and, consequently, more amenable to IL-12 gene therapy *in vivo*. Using the gene gun, we first demonstrated that transfection of 4T1 cells with IFNγ DNA resulted in enhanced MHC class I expression. Furthermore, in a conventional vaccination-challenge experiment, we were able to show the increased immunogenicity of IFNγ gene-transfected 4T1 cells as compared with the non-transfected 4T1 cells or Luciferase gene-transfected cells (**Figure 2**). To evaluate the potency of our proposed strategy, we are currently applying *in vivo* IL-12 gene treatment to vaccinated 4T1 tumor-bearing mice. We suggest that a combination of *ex vivo* and *in vivo* gene transfer strategies may provide a new approach for tumor immunotherapy that may enhance an immune response via implementation of two complementary mechanisms.

Role of T cells in anti-metastatic effect of IL-12 gene therapy against 4T1 tumors.

Our results described in a previous report showed that T cell-deficient nude mice were able to control metastatic growth of 4T1 tumor following IL-12 gene therapy, suggesting that T cells

don't play a major role in this anti-metastatic event. These results were unexpected, since we have previously reported (8) that the regression of immunogenic tumors induced by the current gene gun-mediated IL-12 gene therapy protocol is CD8+ T cell-dependent. In an attempt to confirm the results obtained with nude mice, we performed a series of T-cell depletion experiments in which 4T1 tumor-bearing mice were treated with either: (a) Luc cDNA, (b) IL-12 cDNA plus control rat IgG, or (c) IL-12 cDNA plus anti CD4/CD8 mAb. The results in **Figure 3** demonstrate that mice depleted of T cells were still able to suppress metastases in the lungs following IL-12 gene therapy, conforming the results previously obtained previously with nude mice. Together, these results strongly suggest that T cells do not mediate the IL-12-induced anti-metastatic effect against the 4T1 tumor.

Role of NK cells and IFNγ in anti-metastatic effect of IL-12 gene therapy against 4T1 tumors.

Since T cells do not seem to be involved in the observed systemic anti-metastatic effect of IL-12 gene therapy in the 4T1 model, and because IL-12 protein did not exhibit a direct inhibitory effect on the growth of 4T1 tumor *in vitro* (data not shown), we suggested that transgenic IL-12 may have acted by stimulating or augmenting some local or regional host defense mechanisms that could suppress metastasis of 4T1 tumor cells. It has been previously shown that, following a subcutaneous implantation, 4T1 tumor cells metastasize into the blood and regional lymph nodes and then into the lungs (13). We hypothesized, therefore, that this IL-12 gene therapy protocol may induce an immune or inflammatory reactivity in TDLN which in turn could negatively affect tumor growth, invasion, or subsequent spread to the lungs. To test this possibility, we decided to evaluate NK cell activity in TDLN cells following IL-12 gene therapy, because it has been reported previously that IL-12 augments both the cytotoxicity of NK cells (20) and the IFNγ production by NK cells (20,21). We have indeed previously shown, as described in the previous Annual Report, that IL-12 gene therapy results in an augmented IFNγ production in TDLN.

Mice were injected i.d. in the middle of the abdominal area with 10^5 4T1 tumor cells, and skin-transfected with IL-12 cDNA or Luc cDNA 7 and 10 days later. On day 11, axillary lymph nodes were removed, and the derived cells were either directly assayed for NK cytotoxicity against YAC-1 cells, or incubated *in vitro* without any stimulation at 37°C for 48 hr, followed by assaying of supernatants for IFNγ by ELISA. The results in **Figure 4A** show that NK activity in TDLN of IL-12 gene-treated mice was higher than in control mice. Similarly, the results presented in **Figure 4B** show that IL-12 gene therapy caused a substantially enhanced production of IFNγ in TDLN as compared with mice treated with Luc cDNA, or untreated tumor-bearing mice. In addition, it was found that recombinant mIFNγ at doses equal or above 1 ng/ml could significantly inhibit proliferation of 4T1 cells *in vitro* (data not shown).

Because we found increased activity of NK cells TDLN from mice that received IL-12 gene therapy, we next tested whether NK cells were involved in the IL-12-induced anti-metastic effect against 4T1 tumors. To answer this question, mice received injections of anti asialo GM1 Ab, starting one day before the treatment with IL-12, and continued throughout the

treatment. The results in **Figure 5** show that the anti-metastatic effect of IL-12 gene therapy was reduced in NK cell-depleted mice, suggesting a role for NK cells in IL-12-induced anti-metastatic effect against 4T1 tumor. Experiments on neutralization of endogenous IFNγ in vivo are currently being performed.

Localized IL-12 gene delivery resulted in a systemic effect against a solid secondary 4T1 tumor.

The mechanism of the observed anti-metastatic effect of IL-12 gene therapy against 4T1 tumor is not clear. Since it appears that T cells don't play a role in the observed systemic anti-metastatic effect of IL-12 gene therapy, and because IL-12 protein did not exhibit any inhibitory effect on the growth of 4T1 tumor *in vitro* (data not shown), it is possible that transgenic IL-12 may stimulate or augment some local or regional nonspecific host defense mechanisms that can suppress 4T1 tumor metastasis into the lungs. Alternatively, it is possible that IL-12 gene therapy results in a systemic activation of certain defense mechanism(s). To test this possibility, 4T1 tumor-bearing mice were implanted intradermally with a secondary 4T1 tumor on the back, one day after the beginning of treatment of the primary, abdominal tumor with IL-12 cDNA. The growth of both primary and secondary tumor was followed. The results presented in **Figure 6** show that the localized IL-12 gene delivery resulted in a systemic antitumor effect, reducing growth of a secondary intradermal tumor. These findings are in agreement with our data obtained from studies on systemic anti-metastatic effect of IL-12 gene therapy.

Combination of IL-12 with IL-18 gene therapy.

Interleukin-18 (IL-18) can be released as a bioactive form from the inactive IL-18 precursor (pro-IL-18) by IL-1β converting enzyme (ICE). Transfection of pro-IL-18 and ICE cDNA results in higher level of IL-18 than that of pro-IL-18 alone *in vitro*. IL-12 and IL-18 protein synergistically induce regression of murine tumor (22). We hypothesized that a combination of IL-12, pro-IL-18 and ICE cDNA could augment antitumor effect by generating elevated level of bioactive IL-18 and IFN-γ. To test this hypothesis, we compared *in vitro* production of IFN-γ from mouse splenocytes stimulated with transfected tumor cells by IFN-γ bioassay and tumor growth with gene gun treatment among (1) control vehicle, (2) IL-12 alone, (3) IL-12 and pro-IL-18, and (4) IL-12, pro-IL-18 and ICE. Each cytokine cDNA in a eukaryotic expression vector or its mixture were coated onto gold particles and delivered *in vitro* into tumor cells or *in vivo* into established viable tumor and surrounding skin tissue by gene gun technique. The combination of IL-12, pro-IL-18 and ICE cDNA induced the highest level of IFN-γ from splenocytes *in vitro* (**Figure 7**). Similarly, its transfer into the skin overlying an interdermal tumor resulted in the hishest level of IFN-γ at the skin tissue treatment site. Furthermore, in the gene gun-treatment model, the combinatorial therapy of IL-12, pro-IL-18 and ICE cDNA was the most effective among all tested groups in suppressing of TS/A tumor growth (**Figure 8**), and 47 % of mice achieved complete regression (**Table 1**). Our preliminary data suggest that the antitumor effect was abrogated when $CD8^+$ T cells were depleted, and moderately suppressed by neutralization of IFN-γ. These results indicate that a combinatorial gene therapy with IL-12, pro-IL-18 and ICE cDNA may have a synergistic antitumor effect against certain breast tumors.

Combination of IL-12 gene therapy with chemokine gene therapy.

In an attempt to increase the efficacy of IL-12 gene therapy, we combined it with two novel chemokine genes, namely IP-10 and lymphotactin (Lptn), known to induce antitumor effects in murine models (23,24) The results of two representative experiments are shown in **Figure 9**. In first experiment, the MethA sarcoma tumor model was used, in which IL-12 alone achived a moderate antitumor effect (1 of 5 mice rejected the tumor). Addition of IP-10 gene resulted in suppression of IL-12-induced antitumor effect, whereas Lptn acted synergistically with IL-12, achieving tumor regression in 4 of 5 mice (Figure 9A). However, this synergistic effect of IL-12 and Lptn could not been reproduced in following experiments, either with MethA tumor, or TS/A tumor model (Figure 9B). The suppressive effect of IP-10 was quite reproducible (data not shown).

Development of a novel genetic vaccine approach for cancer therapy.

In parallel with the experiments described above, we are investigating a separate approach for cancer gene immunotherapy, in which mice are vaccinated with a gene coding for tumor-associated antigen. In case with weakly immunogenic tumors, such as 4T1 adenocarcinoma, genetic vaccines may have a potential to induce T cell immune response. As a model tumor antigen, we have chosen the melanoma-associated antigen gp100. A weakly immunogenic murine B16 melanoma stably transfected with human gp100 cDNA was used as a tumor model. Particle-mediated delivery of 2.5 μgs of human gp100 gene into the skin of mice, followed by tumor challenge, resulted in complete tumor protection in 40% of mice. Importantly, delivery of granulocyte macrophage colony-stimulating factor (GM-CSF) DNA together with gp100 DNA resulted in tumor protection in 80-100% of mice (**Figure 10**). The mechanisms of this antitumor effect are being investigated. Potential effects of IL-12 and IL-12 + GM-CSF as adjuvant DNAs for this tumor vaccine approach will be evaluated.

B3. Discussion

The leading cause of death of women with breast cancer is tumor metastases in visceral organs (1-3). Recent progress in tumor and cellular immunology has provided good evidence that human breast tumors express tumor associated antigens (TAA), such as HER-2/neu (25), p53 (26) and DF3/MUC-1 (27), which are capable of inducing cytotoxic T cell responses in patients (28). As a result, immunotherapeutic strategies for treatment of metastatic breast cancers have received increased attention in recent years. One such approach involves the use of recombinant cytokines with the purpose of boosting the existing antitumor immune response. Studies in animal models have shown that IL-12, when compared with other cytokines, has outstanding antitumor efficacy (4,5,9), as was expected based on its known immune stimulatory effects on Th1 cells, cytotoxic T-cells and natural killer (NK) cells (29). Based on these studies, clinical trials with IL-12 were initiated. Unfortunately, the first clinical trial protocol revealed substantial toxicity of recombinant IL-12 protein in humans, resulting in the death of two patients and severe side effects in 15 others (7). Although the lack of a pre-dosing schedule was suggested as the cause for this toxicity, recent experiments have also suggested a need to explore alternative IL-12 delivery mechanisms, to assure treatment that is both safe and effective (30). Using an IL-12 gene therapy approach, we have shown that localized *in vivo* IL-12 gene transfer into skin tissue can result in eradication of established murine tumors and their metastases, leading to the generation of a strong tumor-specific immunological memory (8). More importantly, no signs of IL-12 toxicity were observed following this gene gun-mediated therapy protocol (9). Therefore, we suggested that the gene gun-mediated IL-12 gene therapy protocol may be effective in treatment of metastatic breast cancer.

The results of this study, obtained so far, show that, indeed, *in vivo* gene therapy with IL-12 can result in complete regression of in 50% of cases of the immunogenic, metastatic murine TS/A adenocarcinoma growing intradermally. When a weakly immunogenic, metastatic 4T1 tumor was employed in similar experiments, the growth of intradermal tumors was not affected, but a significant reduction of lung metastases was observed in 4T1 tumor-bearing mice as a result of this IL-12 gene therapy protocol.

The understanding of tumor immunogenicity is crucially important for developing rational design of cancer immunotherapy in humans. The murine tumors used in this study, TS/A and 4T1 mammary adenocarcinomas, were tested for immunogenicity during the first year of study; we found the TS/A tumor to be immunogenic, and 4T1 to be non-immunogenic based on the ability of tumor vaccine induce a protection against a single-dose tumor challenge. In the classical animal studies, a tumor is considered to be immunogenic if it induces, following either surgical excision or vaccination, protection against a secondary tumor challenge *in vivo* (31). However, tumors originally classified as "non-immunogenic" by this criterion, after being biologically or transgenically modified, were often able to induce an immune response even against the unmodified tumor cells (32-35). We suggested then that a tumor which is considered to be non-immunogenic based on immunization-challenge experiments, such as the 4T1 adenocarcinoma employed in our study, may be later characterized as poorly immunogenic if tumor cells, or the experiment conditions, could be modified to induce an immune response. Indeed, by reducing the dose of tumor challenge we were able to demonstrate a low level protection against 4T1 tumors

in vaccinated mice (Figure 1). In addition, we found that TS/A tumor-vaccinated mice were able to suppress growth of 4T1 tumor challenge as compared with naive mice (data not shown). It is evident, therefore, that under certain conditions it may be possible to induce immune response against 4T1 tumor.

Based on these results, we are currently investigating a strategy that is designed to generate a T cell-mediated immune response against the 4T1 tumor. To increase immunogenicity of 4T1 tumor cells, we transfected them *in vitro* with IFNγ cDNA using gene gun. When injected in mice, 4T1-IFNγ tumor cell vaccine resulted in stronger induction of antitumor immunity than a nonmodified 4T1 vaccine, or sham-transfected 4T1 vaccine (Figure 2). We are planning in future study to use this vaccination approach in combination with the current IL-12 gene therapy protocol in hopes of generating even a stronger anti-metastatic effect against 4T1 tumor. As we discuss below, IL-12 gene therapy alone appears to be not strong enough to augment or induce a therapeutic level of T cell immunity.

Even though 4T1 tumor may induce a low level immune response, we show in this study that the anti-metastatic effect against the 4T1 tumor is not T cell-mediated (Figure 3). This suggests that IL-12 fails to induce a therapeutic level of T cell immunity against a weakly immunogenic tumor. These data are in contrast with the T cell-dependent antitumor effect of IL-12 gene therapy against immunogenic tumors (8), but are in agreement with the studies using some other cytokine gene therapy strategies, which showed that the antitumor responses in a number of cases may not involve T cells (33).

The mechanisms of the anti-metastatic effect of IL-12 gene therapy in the 4T1 tumor model are not clear at this time. It appears that T cell activities including T cell-mediated cytotoxicity are not involved, since an anti-metastatic effect of similar magnitude was also observed in T cell deficient (both nude and T cell-depleted) mice. Knowing that 4T1 cells metastasize from a subcutaneous deposit first to TDLN and then into lungs and other organs (13), we hypothesized that some local or regional processes, induced by transgenic IL-12, may be responsible for the reduction of tumor metastasis into the lungs. Indeed, we found an increased NK activity and IFNγ production in TDLN of IL-12-treated mice (Figure 4). This is in agreement with reports of others showing that NK cells and IFNγ are induced by IL-12 (5,20,21,29) and can confer direct cytotoxicity to tumor cells (20,21). These results suggest that the immune activation in TDLN induced by IL-12 gene therapy may restrict or inhibit tumor metastasis from the primary tumor site into the lungs. The role of IFNγ in controlling 4T1 tumor metastases *in vivo* is currently under investigation.

Our results show that systemic effect of IL-12 gene therapy can be detected not only against metastases in the lungs, but also against secondary intradermal tumors (Figure 6). It would be interesting to know whether both of these secondary tumors are controlled by the same or different mechanisms. It is possible, for example, that although T cells are not crucial against lung metastases, they may play a role in suppression of intradermal secondary 4T1 tumors. We are planning to perform in vivo depletion experiments and immunohistological studies to address these questions.

In order to augment the antitumor effect of IL-12 gene therapy, we attempted to combine IL-12 gene therapy with other immunomodulatory genes. The combination of IL-12, pro-IL-18 and ICE cDNA induced the highest level of IFN-γ from splenocytes *in vitro* (Figure 7) and was the most effective among all groups to suppress the TS/A tumor growth (Figure 8, Table 1). These results are in agreement with the study showing a synergistic antitumor effect of IL-12 and IL-18 (22). Combination of IL-12 cDNA with IP-10 or Lymphotactin cDNA proved to be less effective (Figure 9). Our specific strategy for future research is to increase the anti-tumor immune response by combining the IL-12 gene either with other immunomodulatory (e.g.,CD40, CD40L) genes *in vivo*, or with other agents known to induce immune response (e.g., IL-2-antibody fusion protein, anti-CD3 mAb, anti-CD40 mAb). We also plan to pursue the development of a novel genetic approach for cancer therapy, in which mice are vaccinated with a gene coding for tumor-associated antigen in combination with granulocyte macrophage colony-stimulating factor (GM-CSF) gene. In case with weakly immunogenic tumors, such as 4T1 adenocarcinoma, genetic vaccines may have a potential to induce T cell immune response. Using a model gp100 tumor antigen, we showed that delivery of GM-CSF DNA together with gp100 DNA resulted in the protection in 80-100% of mice from a gp100-expressing tumor (Figure 10). The mechanisms of this antitumor effect are being investigated. It is possible that the combination of genetic vaccination approach with IL-12 gene therapy approach may result in a synergistic antitumor effect.

Based on this and previous studies (8,9), we suggest that gene gun-mediated *in vivo* IL-12 gene therapy approach may be further developed as an effective and safe alternative to systemic IL-12 protein therapy. We believe that extension of this study may provide an experimental rationale for proceeding to a clinical trial of gene gun-mediated IL-12 gene therapy for breast cancer. In addition, the TS/A and 4T1 mammary tumor cell lines used in this study, and the different antitumor effect of IL-12 gene therapy on primary tumors versus visceral metastases, may provide highly desirable experimental models for immunotherapy and gene therapy studies related to human breast cancer.

B4. Recommendations in Relation to the Statement of Work

The work completed to date strongly supports our expectation that IL-12 gene therapy protocols can be effective against metastatic disease in murine models of mammary cancer. The experiments have been performed according to the time table projected in the original proposal, and have yielded mostly positive and exciting results.

During our experiments, we observed a high degree of variability between individual control mice in terms of their lung weights. Because of this problem, some experiments had to be repeated several times in order to obtain statistically significant data. In an attempt to minimize the observed variations between individual mice, we investigated the dependence of lung weight on the site of intradermal tumor cell injection. The most pronounced metastatic disease in the lungs was observed when the tumor was injected in the upper middle side of abdomen, and the least level of metastases was observed when the mice were injected in the lower side of abdomen. Based on these observations, in the future experiments we will systematically implant 4T1 tumor cells in the upper middle part of abdomen.

We plan to follow the Statement of Work as originally outlined with a number of modifications. Thus, in the coming year, we plan to:

- Determine whether tumor vaccination of mice with IFNγ-transfected 4T1 tumor cells in combination with in vivo IL-12 gene therapy will result in a synergistic antitumor effect.
- Evaluate the role of IFNγ in the observed anti-metastatic effect of IL-12 gene therapy against the 4T1 tumor.
- Evaluate the cellular mechanisms of the observed anti-metastatic effect of IL-12 gene therapy in the lungs using immunohistochemistry.
- Test a combination of IL-12 gene therapy either with other immunomodulatory (CD40, CD40L, gp100) genes *in vivo*, or with other agents known to induce potent immune response (IL-2 fusion protein, anti-CD3 mAb, anti-CD40 mAb).

C. CONCLUSIONS

- 4T1 tumor was re-evaluated and found to be weakly immunogenic rather than non-immunogenic as was previously suggested.
- Transfection of 4T1 cells with IFNγ cDNA results in increased immunogenicity of tumor vaccine
- Anti-metastatic effect induced by IL-12 gene therapy against 4T1 breast tumor is not T cell-mediated, but involves NK cells possibly via IFNγ-related mechanism.
- Localized IL-12 gene delivery can result in a systemic effect not only against lung metastases, but also against solid secondary 4T1 tumors.
- A combinatorial gene therapy with IL-12, pro-IL-18 and ICE cDNA confers a synergistic effect against TS/A breast tumor.
- Combination of IL-12 cDNA with lymphotactin cDNA, but not with IP-10 cDNA, may result in a better antitumor effect of gene therapy.
- A new approach for tumor gene immunotherapy, consisting of combining the cDNA expression vectors encoding for tumor-accociated antigen with GM-CSF cDNA, and delivering into skin using a gene gun, showed a promising antitumor effect.

D. REFERENCES

1. Marshall, E. Search for a Killer: Focus Shifts From Fat to Hormones. Science 259, 618-621 (1993).

2. Shapiro, T. and Clark, P. Breast Cancer: What the Primary Care Provider Needs to Know. Nurse Pract. USA 20, 36, 39-40, 42 (1995).

3. Lippman, M. The Development of Biological Therapies for Breast Cancer. Science 259, 631-632 (1993).

4. Brunda, M., Luistro, L., Warrier, R., Wright, R., Hubbard, B., Murphy, M., Wolf, S. and Gately, M. Antitumor and Antimetastatic Activity of Interleukin 12 against Murine Tumors. J Exp. Med. 178, 1223-1230 (1993).

5. Nastala, C., Edington, H., McKinney, T., Tahara, H., Nalesnik, M., Brunda, M., Gately, M., Wolf, S., Schreiber, R., Storkus, W. and Lotze, M. Recombinant IL-12 Administration Induces Tumor Regression in Association with IFN-g Production. J Immunology 153, 1697-1706 (1994).

6. Orange, J., Salazar-Mather, T., Opal, S., Spencer, R., Miller, A., McEwen, B. and Biron, C. Mechanism of Interleukin 12-mediated Toxicities during Experimental Viral Infections: Role of Tumor Necrosis Factor and Glucocorticoids. J. Exp. Med. 181, 901-914 (1995).

7. Marshall, E. Cancer Trial of Interleukin-12 Halted. Science 268, 1555 (1995).

8. Rakhmilevich, A.L. *et al.* Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors. Proc. Natl. Acad. Sci. USA 93, 6291-6296 (1996).

9. Rakhmilevich, A.L., Janssen, K., Turner, J., Culp, J. & Yang, N.-S. Cytokine gene therapy of cancer using gene gun technology: Superior antitumor activity of IL-12. Human Gene Therapy 8, 1303-1311 (1997).

10. Noguchi, Y., Chen, Y.N. and Old, L. A Mouse Mutant p53 Product Recognized by CD4+ and CD8+ T cells. Proc. Natl. Acad. Sci. USA 91, 3171-3175 (1994).

11. Nanni, P., De Giovanni, C., Lollini, P.-L., Nicoletti, G. and Prodi, G. TS/A: a New Metastasizing Cell Line from a BALB/c Spontaneous Mammary Adenocarcinoma. Clin. Exp. Metast. 1, 373-380 (1983).

12. Cavallo, F., Di Pierro, F., Giovarelli, M., Gulino, A., Vacca, A., Stoppacciaro, A., Forni, M., Modesti, A. and Forni, G. Protective and Curative Potential of Vaccination with Interleukin-2

Gene-transfected Cells from a Spontaneous Mouse Mammary Adenocarcinoma. Cancer Res. 53, 5067-5070 (1993).

13. Aslakson, C. and Miller, F. Selective Events in the Metastatic Process Defined by Analysis of the Sequential Dissemination of Subpopulations of a Mouse Mammary Tumor. Cancer Res. 52, 1399-1405 (1992).

14. Sotomayor, E., Fu, Y.X., Lopez-Cepero, M., Herbert, L., Jimenez, J., Albarracin, C. and Lopez, D. Role of Tumor-Derived Cytokines on the Immune System of Mice Bearing a Mammary Adenocarcinoma. J. Immunology 147, 2816-2823 (1991).

15. Hossain, A., Sarkar, A. and Sarkar, N. Mixed Inocula of Mouse Mammary Tumour Cell Subpopulations Result in Changes of Organ-Specific Metatasis. Clin. Expl. Met. 9, 6, 501-515 (1991).

16. Lollini, P. L., Bosco, M., Cavallo, F., De Giovanni, C., Giovarelli, M., Landuzzi, L., Musiani, P., Modesti, A., Nicoletti, G., Palmieri, G., Santoni, A., Young, H., Forni, G. and Nanni, P. Inhibition of Tumor Growth and Enhancement of Metastasis after Transfection of the g-Interferon Gene. Int. J. Cancer 55, 320-329 (1993).

17. Pericle, F., Giovarelli, M., Colombo, M., Ferrari, G., Musiani, P., Modesti, A., Cavallo, F., Di Pierro, F., Novelli, F. and Forni, G. An Efficient Th2-Type Memory Follows CD8+ Lymphocyte-Driven and Eosinophil-Mediated Rejection of a Spontaneous Mouse Mammary Adenocarcinoma Engineered to Release IL-4. J. Immunology 153, 5659-5673 (1994).

18. Apostolopoulos, V., McKenzi, I.F.C., and Pietersz, A. Breast cancer immunotherapy: current status and future prospects. Immunology and Cell Biology 74, 457-464 (1996).

19. Alllione, A., Consalvo, M., Nanni, P., Lollini, P.L., Cavallo, F., Giovarelli, M., Forni, M., Gulino, A., Colombo, M.P., Dellabona, P., Hock, H., Blankenstein, T., Rosenthal, F.M., Gansbacher, B., Bosco, M.C., Musso, T., Gusella, L. and Forni, G. Immunizing and curative potential of replicating and nonreplicating murine mammary adenocarcinoma cells engineered with interleukin (IL)-2, IL-4, IL-6, IL-7, IL-10, tumor necrosis factor , granulocyte-macrophage colony stimulating factor, and -Interferon gene or admixed with conventional adjuvants. Cancer Research 54, 6022-6026 (1994).

20. Gately, M., Warrier, R., Honasoge, S., Carvajal, D., Faherty, D., Connaughton, S., Anderson, T., Sarmiento, U., Hubbard, B. and Murphy, M. Administration of Recombinant IL-12 to Normal Mice Enhances Cytolytic Lymphocyte Activity and Induces Production of IFN-γ in vivo. International Immunology 6, 157-167 (1994).

21. Brunda, M.J. *et al.* Role of interferon-g in mediating the antitumor efficacy of interleukin-12. J. Immunother. 17, 71-77 (1995).

22. Coughlin C.M., Salhany K.E., Wysocka M. et al. Interleukin-12 and Interleukin-18

synergistically induce murine tumor regression which involves inhibition of angiogenesis. J.Clin.Invest., 101 (6):1441-1452 (1998).

23. Luster A.D. and Leder P. IP-10, a -C-X-C- chemokine, elicits a potent thymus-dependent antitumor response in vivo. J.Exp.Med. 178:1057-1065 (1993).

24. Dilloo D., Bacon K., Holden W. et al. Combined chemokine and cytokine gene transfer enhances antitumor immunity. Nature medicine, 2(10): 1090-1095 (1996).

25. Disis, M.L., Calenoff, E., McLaughlin, G., Murphy, A.E., Chen, W., Groner, B., Jeschke, M., Lydon, N., McGlynn, E., and Livingston, R.B. et al. Existent T-cell and antibody immuntiy to HER-2/neu protein in patients with breast cancer. Cancer Research 54(1), 16-20 (1994).

26. Tilkin, A.F., Lubin, R., Soussi, T., Lazar, V., Janin, N., Mathieu, M.C., Lefrere, I., Carlu, C., Roy, M., and Kayibanda et al. Primary proliferative T cell response to wild-type p53 protein in patients with breast cancer. European Journal of Immunology 25(6), 1765-1769 (1995).

27. Gimmi, C.D., Morrison, B.W., Mainprice, B.A., Gribben, J.G., Boussiotis, V.A., Freeman, G.J., Park, S.Y., Watanabe, M., Gong, J., Hayes, D.F., Kufe, D.W., and Nadler, L.M. Breast cancer-associated antigen, DF3/MUCi, induces apoptosis of activated human T cells. Nature Medicine 2(12), 1367-70 (1996).

28. Jerome, K.R., Domenech, N. and Finn, O.J. Tumor-specific cytotoxic T cell clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. The Journal of Immunology 151, 1654-1662 (1993).

29. Brunda, M.J. & Gately, M.K. Interleukin-12: Potential role in cancer therapy. *Important Adv. Oncology* , 3-18 (1995).

30. Coughlin, C.M., Wysocka, M., Trinchieri, G., and Lee, W.M.F. The effect of interleukin 12 desensitization on the antitumor efficacy of recombinant interleukin 12. Cancer Research 57, 2460-2467 (1997).

31. Middle, J.G. and Embleton, M.J. Naturally arising tumors of the inbred WAB/Not rat strain. JNCI 67 (3), 637-643 (1981).

32. Gansbacher, B., Zier, K., Daniels, B., Cronin, K., Bannerji, R. and Gilboa, E. Interleukin 2 Gene Transfer into Tumor Cells Abrogates Tumorigenicity and Induces Protective Immunity. J Exp. Med. 172, 1217-1224 (1990).

33. Golumbek, P., Lazenby, A., Levitsky, H., et al. Treatment of Established Renal Cancer by Tumor Cells Engineered to Secrete Interleukin-4. Science 254, 713-716 (1991).

34. Pardoll, D. New strategies for active immunotherapy with genetically engineered tumor cells.

Current Opinion in Immunol. 4, 619-623 (1992).

35. Colombo, M.P., Modesti, A., Parmiani, G. and Forni, G. Local cytokine availability elicits tumor rejection and systemic immunity through granulocyte-T-lymphocyte cross-talk. Cancer Res. 52, 4853-4857 (1992).

PUBLICATIONS AND PRESENTATIONS

1. A.L.Rakhmilevich, K.Janssen and N-S. Yang. Gene gun-mediated IL-12 gene therapy for breast cancer in mice. Cancer Gene Therapy, 4:S52-S53, 1997. A poster presentation at 6th International Conference on Gene Therapy of Cancer, Nov 20-22, 1997, San Diego, CA.

2. N-S. Yang, A.L.Rakhmilevich, P.M.Sondel, D.Mahvi, G.Hogge, and G.MacEwen. Cytokine gene therapy for cancer and DNA cancer vaccines: A gene gun approach. An oral presentation at 1998 Keystone Symposium "Synthetic Non-Viral Gene Delivery Systems", January 19-25, 1998.

3. Rakhmilevich A.L. and Yang N-S. Particle-mediated gene delivery system for cancer research. In *Concepts in Gene Therapy*, editors Strauss and Barranger,Berlin-New York,109-120 1997.

4. Rakhmilevich A.L., Janssen K., Sondel P.M. and Yang N-S. Interleukin 12 gene therapy of a weakly immunogenic mouse mammary carcinoma results in reduction of spontaneous lung metastases via a T cell-independent mechanism (submitted).

5. Rakhmilevich A.L. and Yang N-S. In vivo particle-mediated gene transfer for cancer therapy. In *Gene Therapy of Cancer: Methods and Protocols*, editors Walther and Stein, Berlin (in press).

6. Rakhmilevich A.L., Timmins J.G., Janssen K., Pohlmann E.L., Sheehy M.J. and Yang N-S. Gene gun-mediated IL-12 gene therapy induces antitumor effects in the absence of toxicity: a direct comparison with systemic IL-12 protein therapy (in press).

FIGURE LEGENDS

Figure 1. Immunogenicity of 4T1 adenocarcinomas. This experiment shows that 4T1 tumor is weakly immunogenic. Balb/c mice were injected i.d. on the right side of abdomen with $2x10^6$ gamma-irradiated 4T1tumor cells, or left untreated (no vaccine). Three weeks later, vaccinated mice and naive control mice were challenged i.d. on the left side of abdomen with 10^5 , $5x10^4$, 10^4, or $5x10^3$ replicating 4T1 tumor cells, and tumor growth was followed. Mean tumor diameters $\pm$ SEM are shown for 5 mice per group. A representative of 3 similar experiments is shown.

Figure 2. Increased immunogenicity of IFNγ gene-transfected 4T1 cells. 4T1 tumor cells were gamma-irradiated and immediately transfected in vitro via gene gun with IFNγ plasmid DNA, or control Luciferase (LUC) plasmid DNA, or left nontransfected. Balb/c mice were injected i.d. on the right side of abdomen with $2x10^6$ gamma-irradiated 4T1 tumor cells, or left untreated (no vaccine). Three weeks later, vaccinated mice and naive control mice were challenged i.d. on the left side of abdomen with $5x10^3$ replicating 4T1 tumor cells, and tumor growth was followed. Mean tumor diameters $\pm$ SEM are shown for 5 mice per group on day 21 post tumor challenge.

Figure 3. Anti-metastatic effect of IL-12 gene therapy against 4T1 tumors is not T cell mediated. Balb/c mice were injected i.d. with 10^5 4T1 tumor cells. Skin was transfected with IL-12 cDNA or Luc cDNA on days 7, 10, 13 and 17 post tumor cell implantation. A mixture containing 300 μg of each anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43) was administered intraperitoneally on days 6,11 and 16 (experiment 1), or 6,10,15 and 20 (experiment 2) after tumor implantation. Flow cytometric analysis of peripheral blood cells of these mice, performed 4 days after the last mAb injection, confirmed depletion of 98% of $CD3^+$ cells. Control groups included mice that were treated with the IL-12 cDNA and received rat IgG (Sigma) at the dose 600 μg/mouse per day following the same schedule as the anti-CD4/CD8 mAbs, or mice treated with the Luc cDNA instead of the IL-12 cDNA, or non tumor-bearing naive mice. The lungs were removed on day 31(experiment 1) or 34 (experiment 2), and their weight was determined. Mean lung weight $\pm$ SEM for two combined experiments, where "n" indicates number of mice per group.

Figure 4. Immune activation of TDLN cells following skin transfection with IL-12 DNA. A. Balb/c mice were injected i.d. in the middle of abdomen with 10^5 4T1 tumor cells. On days 7 and 10 post tumor cell implantation, skin overlying the tumor was transfected with 5 μg of IL-12 cDNA or Luc cDNA, or left untreated. On day 11 post tumor cell implantation, axillary lymph nodes were removed, pooled from 3 mice per group, and tested for cytotoxicity against NK-sensitive YAC-1 cells in a 4-hr ^{51}Cr assay. The results are expressed as cytotoxic index at the effector:target ratio 100:1. B. One million of the same lymphoid cells were placed in 1 ml of media for 48 hr. The activity of IFNγ in the supernatants was determined by ELISA. Both graphs depict mean values for two cell samples per group, each sample containing TDLN cells pooled from 3 mice.

carried out. Numbers (8/8, 4/8) indicate mice that rejected tumors relative to total number of mice per group. Data are mean tumor diameters ± SEM for 5-8 mice per group. These results were reproduced in a similar experiment.

Figure 10. Effective vaccination of mice with a gene coding for tumor-associated antigen gp100 in combination with GM-CSF gene. A weakly immunogenic murine B16 melanoma stably transfected with human gp100 cDNA was used as a tumor model. C57Bl/6 mice were vaccinated in the skin by gp100 cDNA alone or in combination with GM-CSF. One week later, vaccinated and naïve mice were challenged i.d. with $5x10^4$ tumor cells, and tumor growth was followed. Particle-mediated delivery of 2.5 μg of human gp100 gene into the skin of mice resulted in complete tumor protection in 40% of mice. Importantly, delivery of GM-CSF cDNA together with gp100 cDNA resulted in tumor protection in 100% of mice in these experiments. The results of two independent experiments, 5 mice per group in each experiment, are presented.

Table 1. Complete regression following gene gun treament.

Treatment	Mice with complete regression	percent (%)
IL-12	4/23	17.4
pro-IL-18	0/15	0
pro-IL-18/ICE	0/15	0
IL-12/pro-IL-18	6/23	26.1
IL-12/pro-IL-18/ICE	11/23	47.8
Control (pNGVL-3)	0/15	0

Gene gun treatment was performed on 6, 8, 10 and 12 days after intradermal injection of 1 x 10^5 TS/A cells. Each treatment, consisted of four transfections with cDNA expression plasmid containing IL-12, pro-IL-18, pro-IL-18/ICE, IL-12/pro-IL-18, or IL-12/pro-IL-18/ICE, or with control plasmid (pNGVL-3). Mice with compele regression means the number of mice in which complete regression of established TS/A tumor was observed following gene gun treatment on day 50 posttumor implantation.

Figure 1

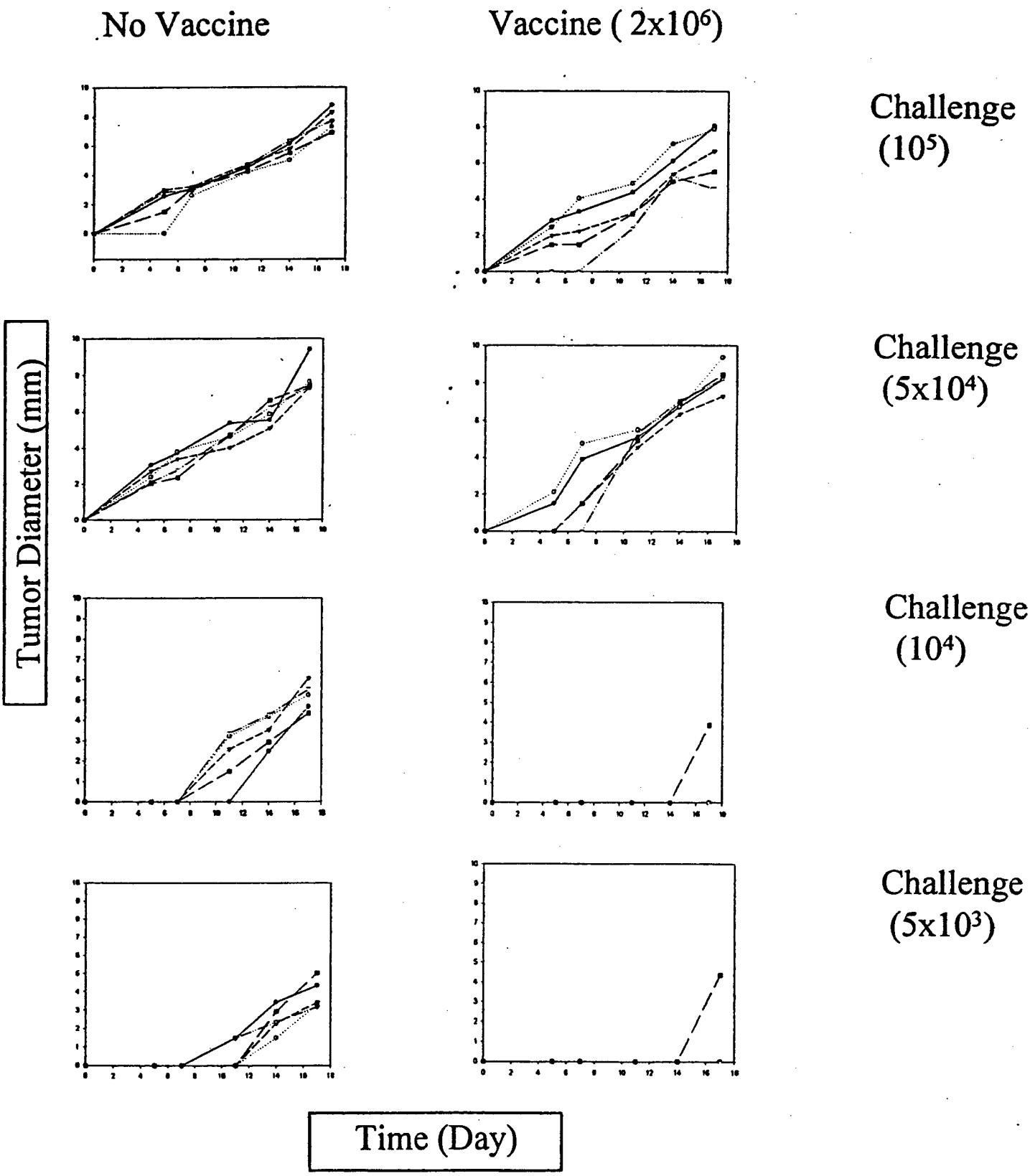

No Vaccine
Vaccine (2x10⁶)
Challenge (10⁵)
Challenge (5x10⁴)
Challenge (10⁴)
Challenge (5x10³)
Tumor Diameter (mm)
Time (Day)

Figure 2

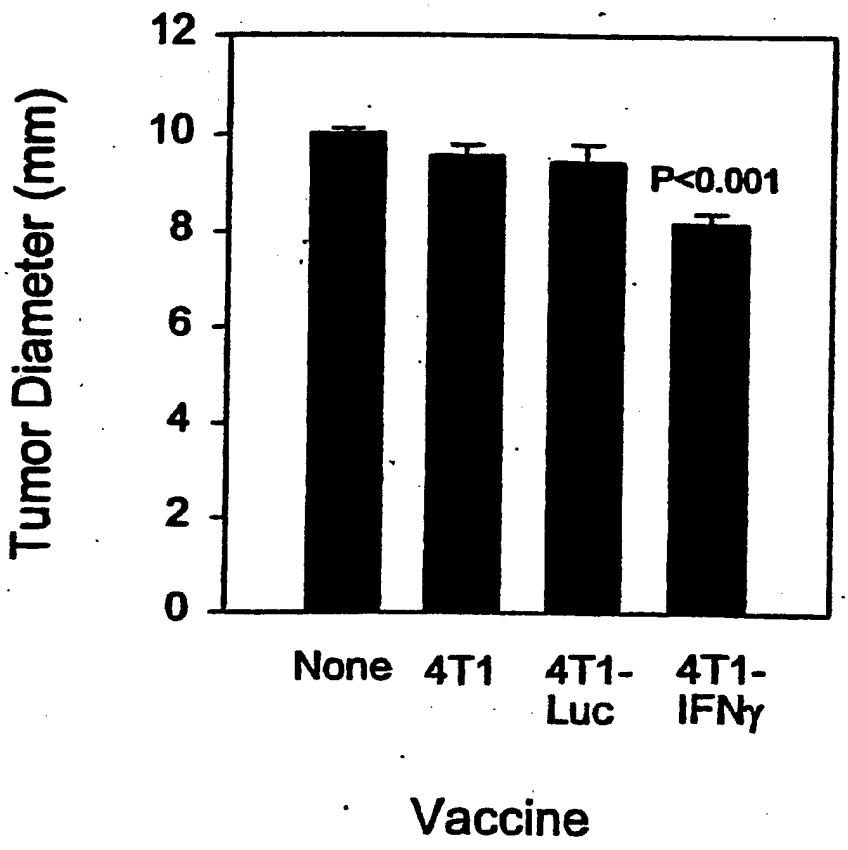
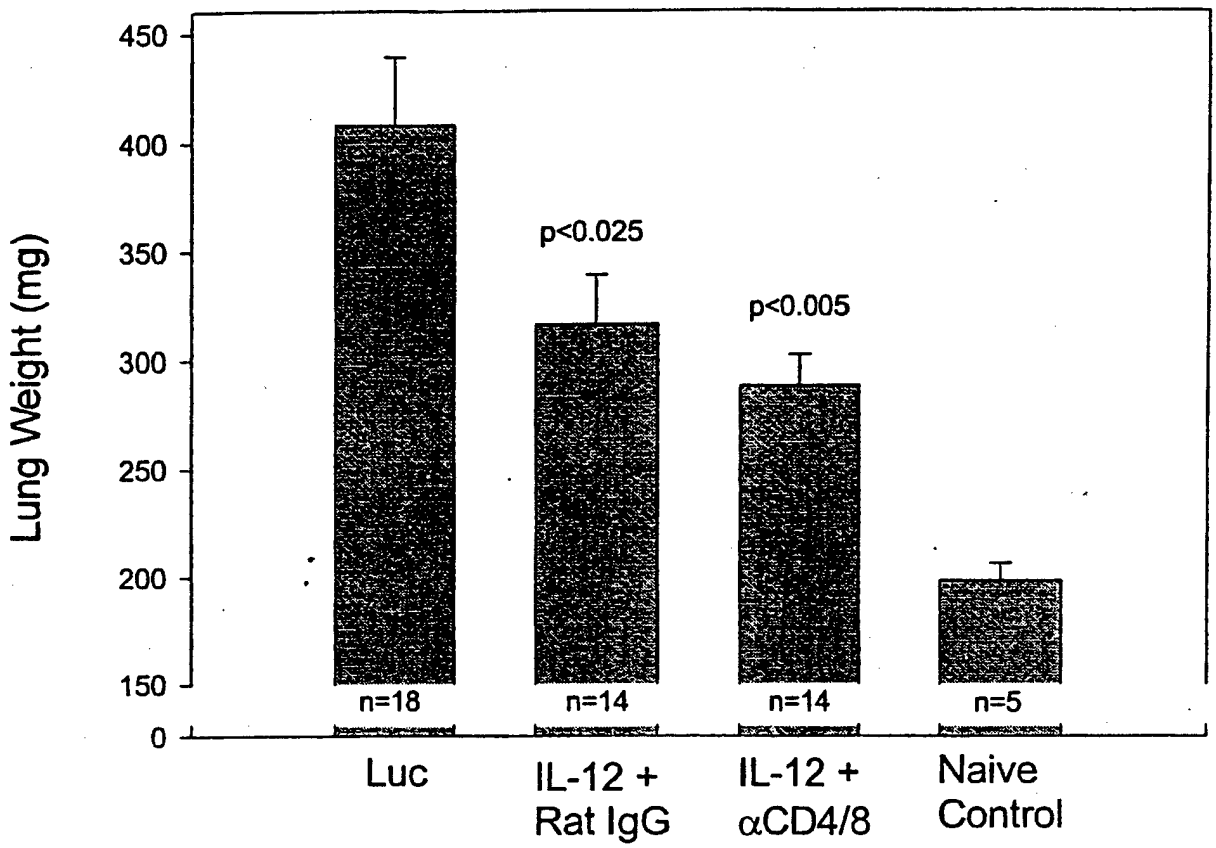

Tumor Diameter (mm)
12
10
8
6
4
2
0
P<0.001
None
4T1
4T1-Luc
4T1-IFNγ
Vaccine

Figure 3


Lung Weight (mg)
450
400
350
300
250
200
150
0
p<0.025
p<0.005
n=18
n=14
n=14
n=5
Luc
IL-12 + Rat IgG
IL-12 + αCD4/8
Naive Control

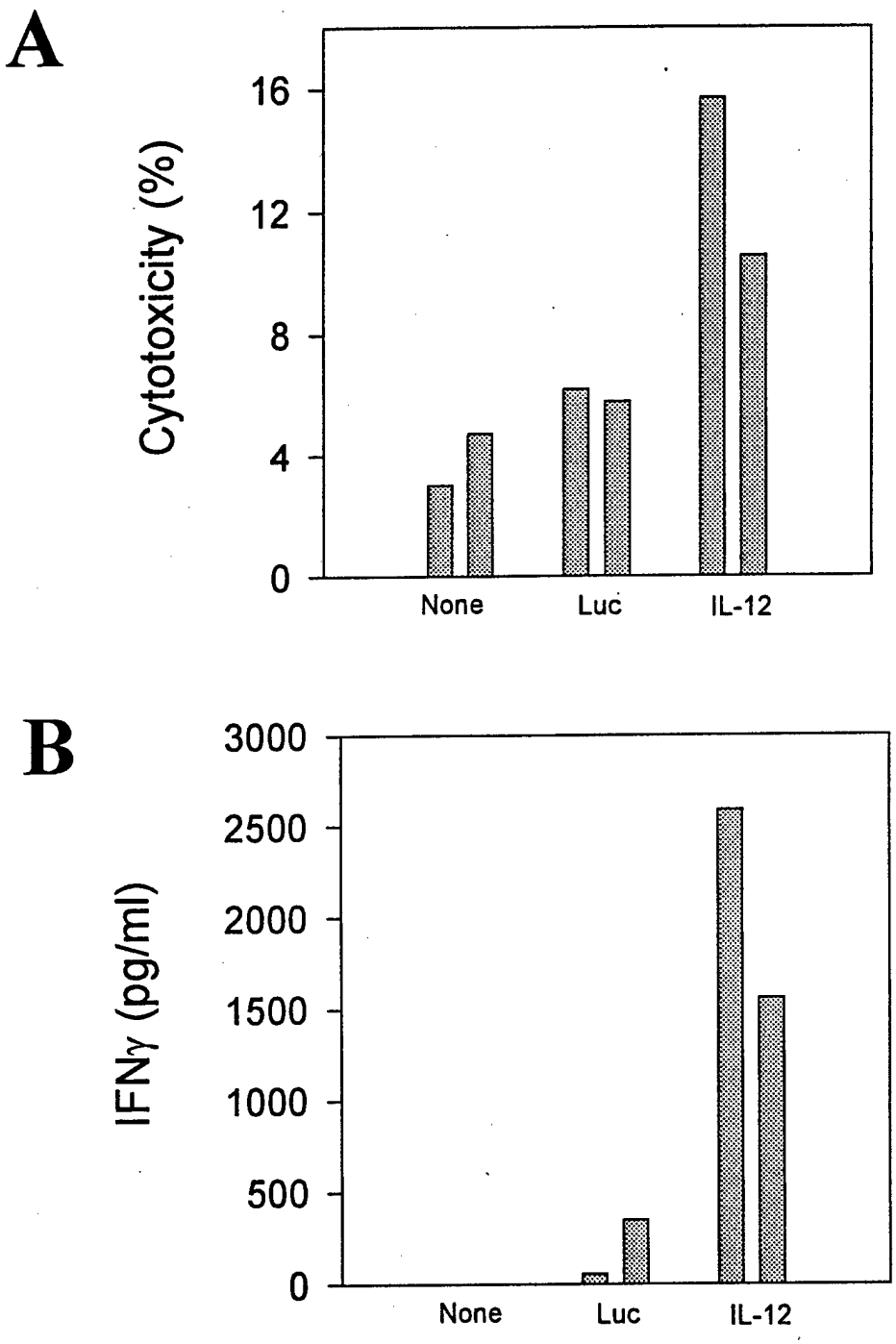

A
Cytotoxicity (%)
16
12
8
4
0
None
Luc
IL-12
B
IFNγ (pg/ml)
3000
2500
2000
1500
1000
500
0
None
Luc
IL-12

Figure 5

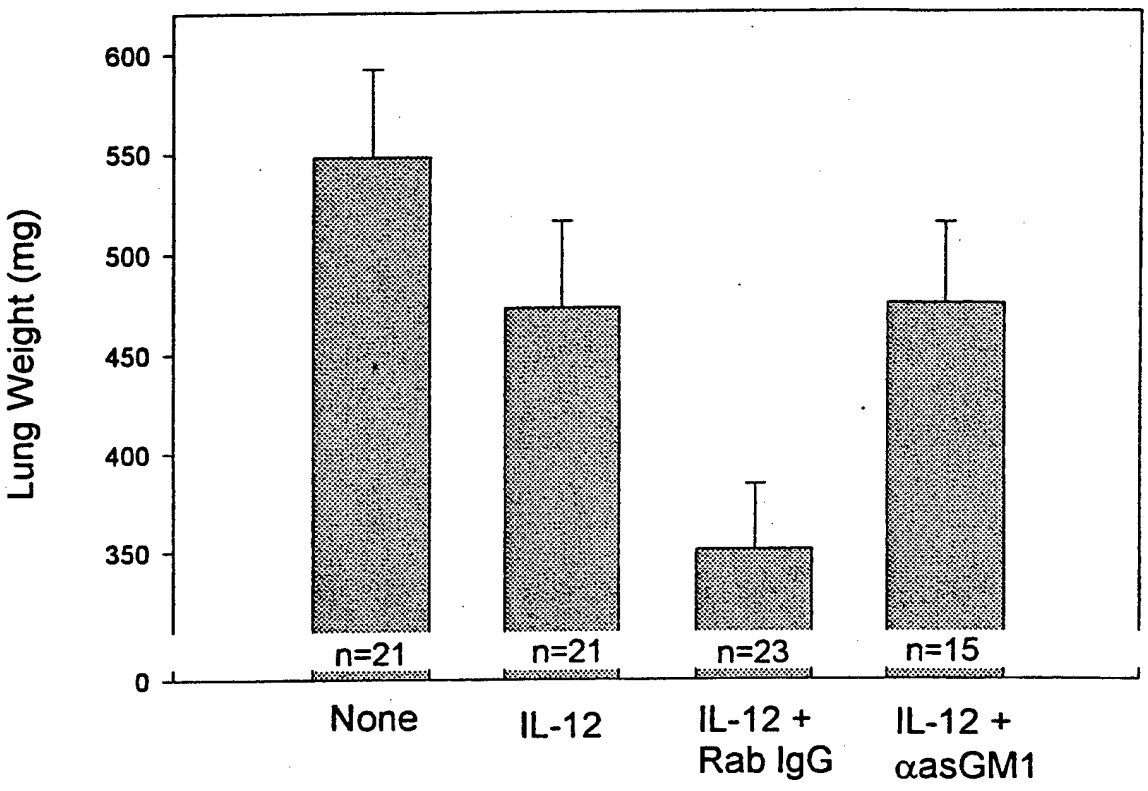
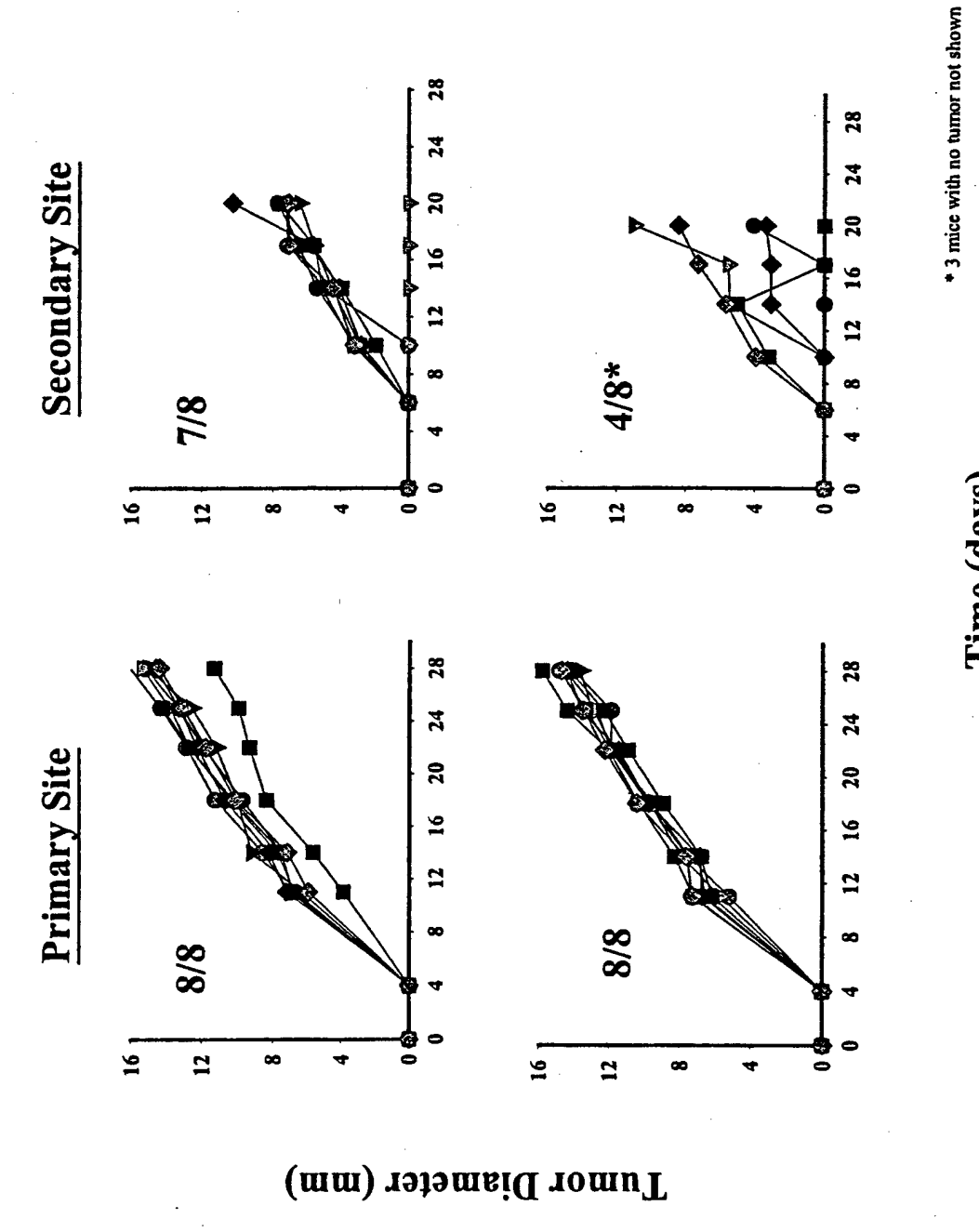

Lung Weight (mg)
600
550
500
450
400
350
0
n=21
n=21
n=23
n=15
None
IL-12
IL-12 + Rab IgG
IL-12 + αasGM1

Figure 6


Primary Site
Secondary Site
Luc
IL-12
8/8
7/8
8/8
4/8*
Tumor Diameter (mm)
Time (days)
* 3 mice with no tumor not shown

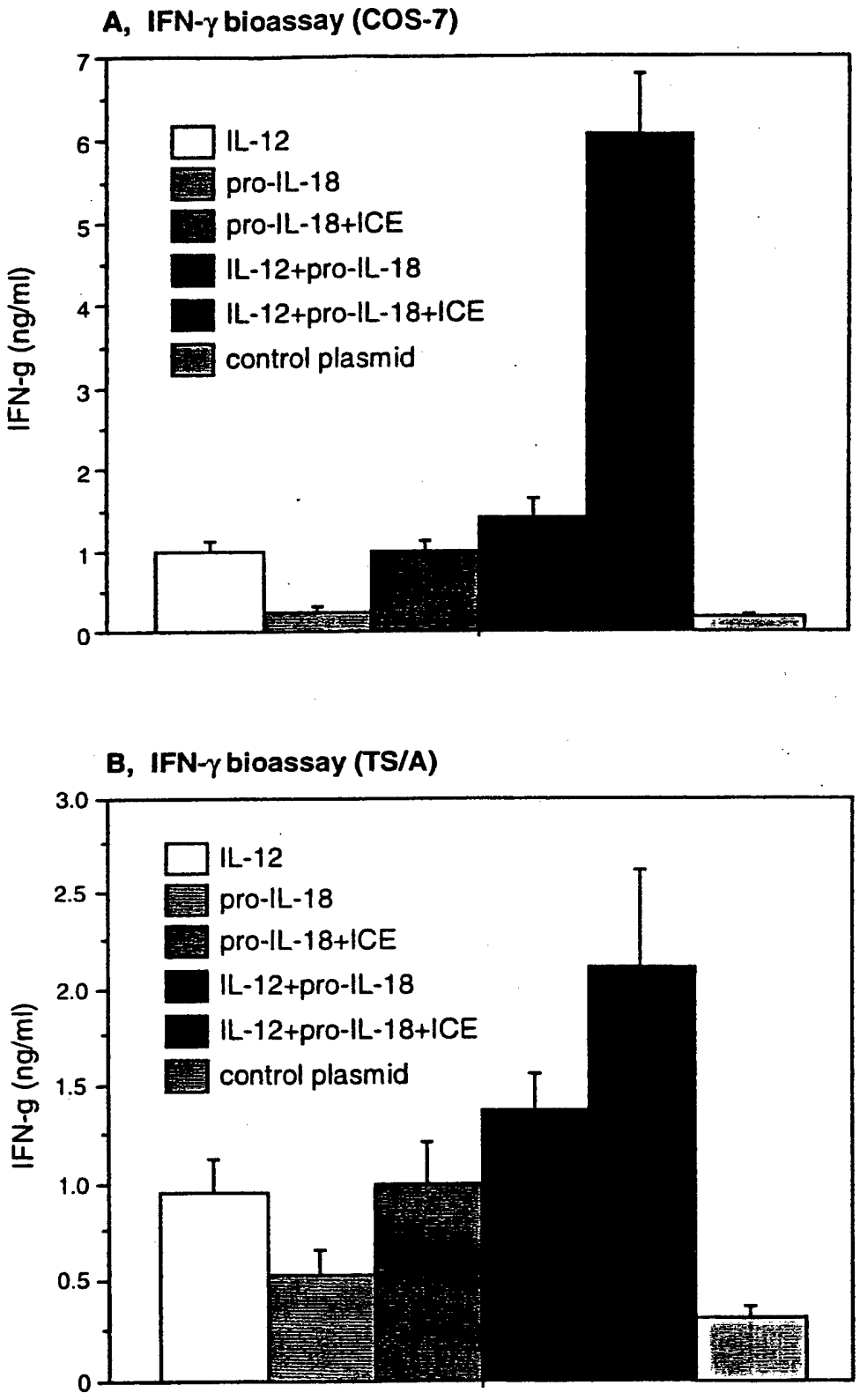

A, IFN-γ bioassay (COS-7)
IFN-g (ng/ml)
0
1
2
3
4
5
6
7
IL-12
pro-IL-18
pro-IL-18+ICE
IL-12+pro-IL-18
IL-12+pro-IL-18+ICE
control plasmid
B, IFN-γ bioassay (TS/A)
IFN-g (ng/ml)
0
0.5
1.0
1.5
2.0
2.5
3.0
IL-12
pro-IL-18
pro-IL-18+ICE
IL-12+pro-IL-18
IL-12+pro-IL-18+ICE
control plasmid

Figure 8

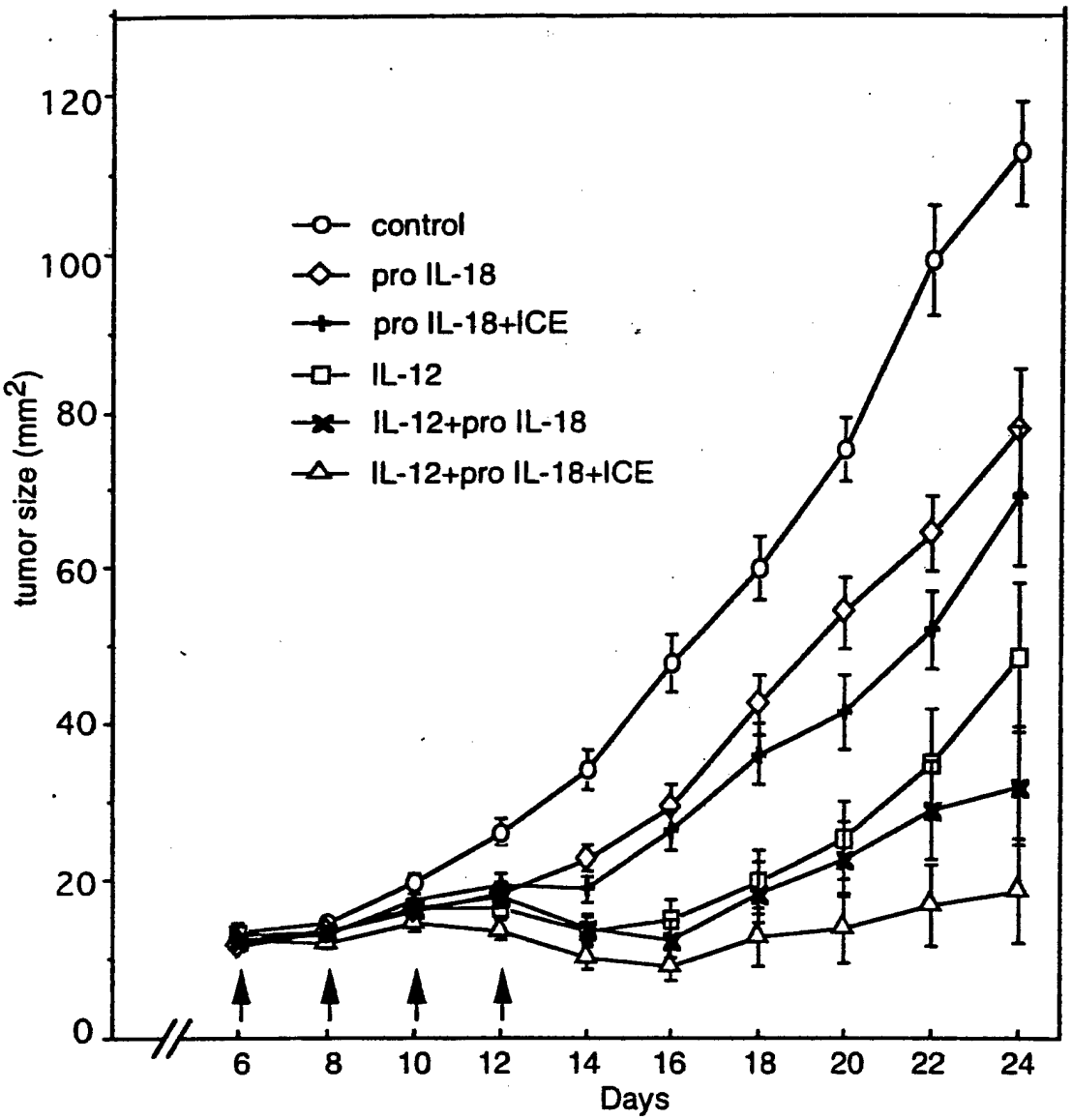
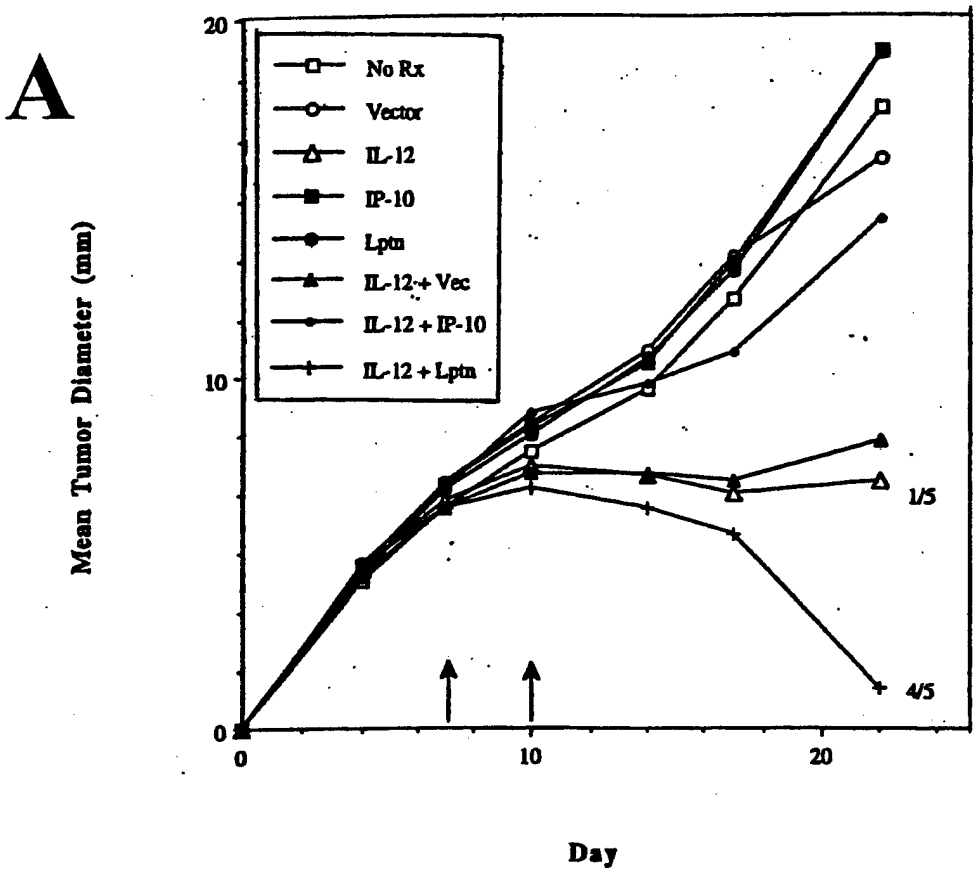

control
pro IL-18
pro IL-18+ICE
IL-12
IL-12+pro IL-18
IL-12+pro IL-18+ICE
tumor size (mm2)
0
20
40
60
80
100
120
6
8
10
12
14
16
18
20
22
24
Days

Figure 9


A
Mean Tumor Diameter (mm)
No Rx
Vector
IL-12
IP-10
Lptn
IL-12 + Vec
IL-12 + IP-10
IL-12 + Lptn
1/5
4/5
Day

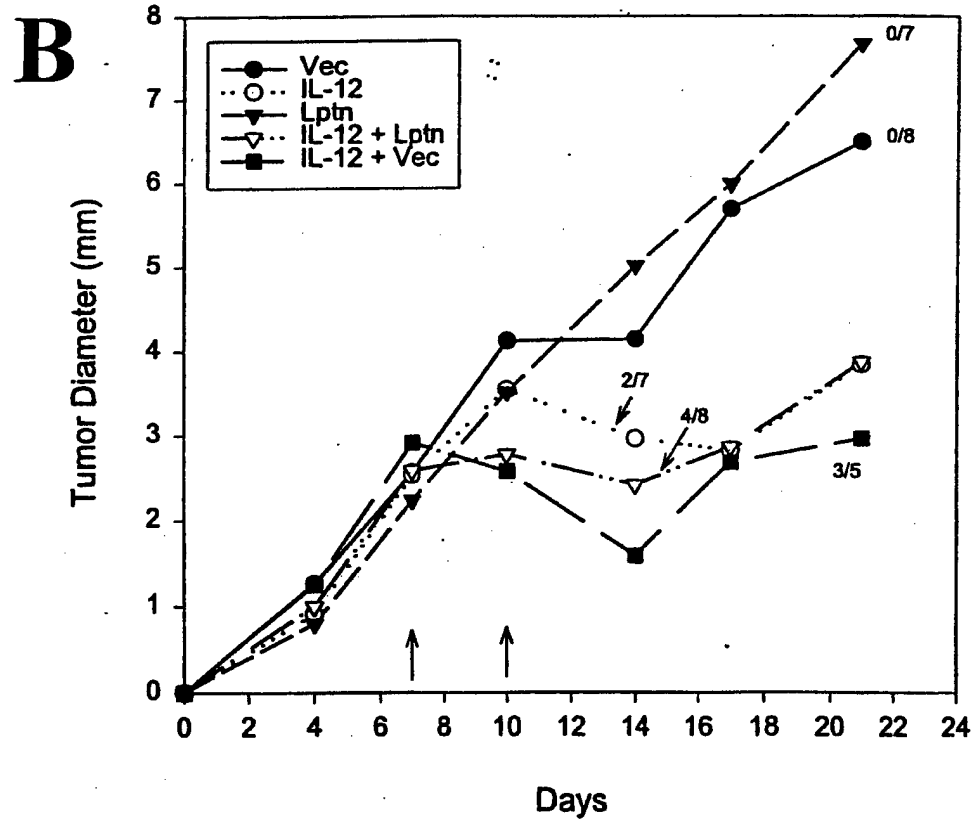
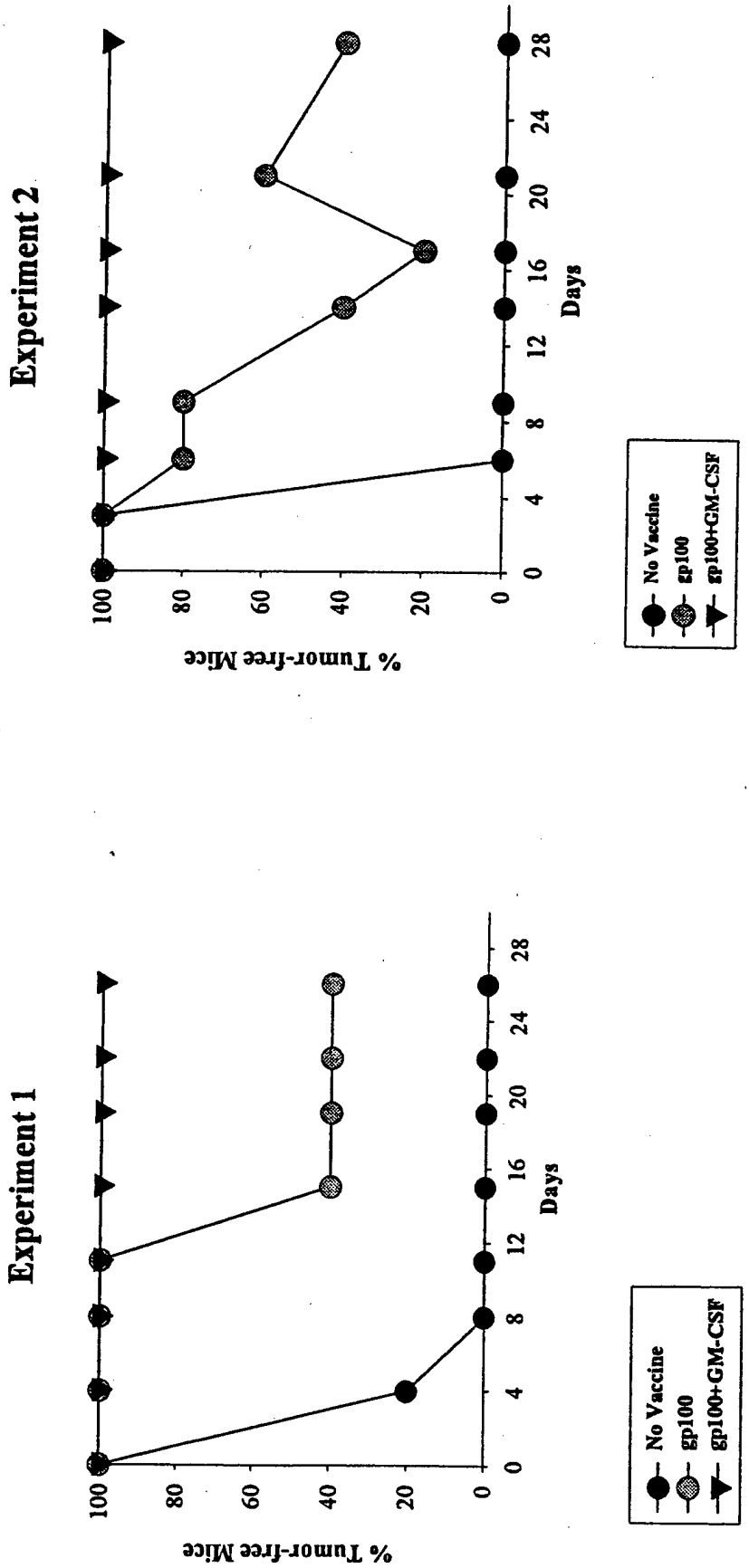

B
Tumor Diameter (mm)
Vec
IL-12
Lptn
IL-12 + Lptn
IL-12 + Vec
0/7
0/8
2/7
4/8
3/5
Days

Figure 10


Experiment 2
% Tumor-free Mice
100
80
60
40
20
0
0
4
8
12
16
20
24
28
Days
No Vaccine
gp100
gp100+GM-CSF
Experiment 1
% Tumor-free Mice
100
80
60
40
20
0
0
4
8
12
16
20
24
28
Days
No Vaccine
gp100
gp100+GM-CSF